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Case Report

Indirect Evidence of TTV Replication in Bone Marrow Cells, But Not in Hepatocytes, of a Subacute Hepatitis/Aplastic Anemia Patient

Kentaro Kikuchi,[1] Hiroshi Miyakawa,[1]* Kazuhiro Abe,[1] Makoto Kako,[1] Kazuhiko Katayama,[2] Shuetsu Fukushi,[2] and Shunji Mishiro [3]

[1]*Fourth Department of Internal Medicine, Teikyo University School of Medicine, Takatsu-ku, Kawasaki, Kanagawa, Japan*
[2]*Basic Research Division, BioMedical Laboratories Inc, Saitama, Japan*
[3]*Department of Medical Sciences, Toshiba General Hospital, Tokyo, Japan*

The presence of a new DNA virus (TTV) has been reported in sera from patients with posttransfusion hepatitis of unknown etiology. The precise replication site of TTV, however, has not been established. In this study, the presence of TTV in liver autopsy material, and in bone marrow biopsy and autopsy samples taken from a subacute hepatitis/aplastic anemia patient was determined by PCR and Southern blot analyses. Liver cells were found to contain only TTV DNA and not mRNA. Bone marrow material, especially that taken at biopsy, contained high levels of TTV DNA. It is suggested that the TTV replication site was in the bone marrow rather than in the liver, and that TTV infection was the cause of this patient's aplastic anemia. The precise etiological association of TTV with hepatitis remains to be established. ***J. Med. Virol. 61:165–170, 2000.***

KEY WORDS: PCR; Southern blot analysis; TTV DNA

INTRODUCTION

The N22 cDNA clone, isolated previously from the serum of a Japanese patient with posttransfusion hepatitis of unknown etiology, has been found to have its origin in the genome of a novel single-stranded DNA virus, designated TT virus (TTV) [Nishizawa et al., 1997]. TTV DNA has been detected by the polymerase chain reaction (PCR) in approximately 40% of Japanese hepatitis cases of unknown etiology [Okamoto et al., 1998]. Investigators in the UK [Simmonds et al., 1998] and in the USA [Charlton et al., 1998] have reported that TTV viremia is seen frequently in patients with chronic liver disease. These and other reports published previously, however, have failed to show a causal association between TTV infection and liver disease [Höhne et al., 1998; Naoumov et al., 1998; Takahashi et al., 1998b; Niel et al., 1999]. Furthermore, evidence that this virus replicates in hepatic cells has yet to be reported.

In the present study, to address this issue, the presence of TTV DNA and mRNA was investigated using liver material obtained at autopsy from a patient with subacute hepatitis/aplastic anemia whose serum was known to contain a high concentration of TTV DNA. The presence of TTV DNA in bone marrow tissue from the same patient was also investigated.

MATERIALS AND METHODS

Patient

A 37-year-old Japanese male with no history of blood transfusion, excessive alcohol intake, drug abuse, nor significant hepatic illness, developed jaundice in July, 1992. The patient was admitted to a nearby hospital on September 17, but no improvement in the jaundice was seen and ascites developed. Upon admission, laboratory tests revealed total bilirubin at 16 mg/dL [normal range: 0.1–1.0], AST 59 IU/L [5–30], ALT 25 IU/L [0–25], and LDH 758 IU/L [203–450]. Aplastic anemia developed and his red blood cell count dropped to 1.71 $\times 10^6/mm^3$, hemoglobin to 6.9 g/dL and hematocrit to 21.7%. Abdominal CT scan showed severe atrophy of liver, splenomegaly, and massive ascites. A diagnosis of subacute hepatitis was made, although tests for viral

The nucleotide sequence data of the TTV isolate reported in this paper will appear in the DDBL, EMBL and GenBank nucleotide sequence databases with association number AB021877.

*Correspondence to: Dr. H. Miyakawa, Fourth Department of Internal Medicine, Teikyo University School of Medicine, 3-8-3 Mizonokuchi, Takatsu-ku, Kawasaki, Kanagawa 213-8507, Japan.

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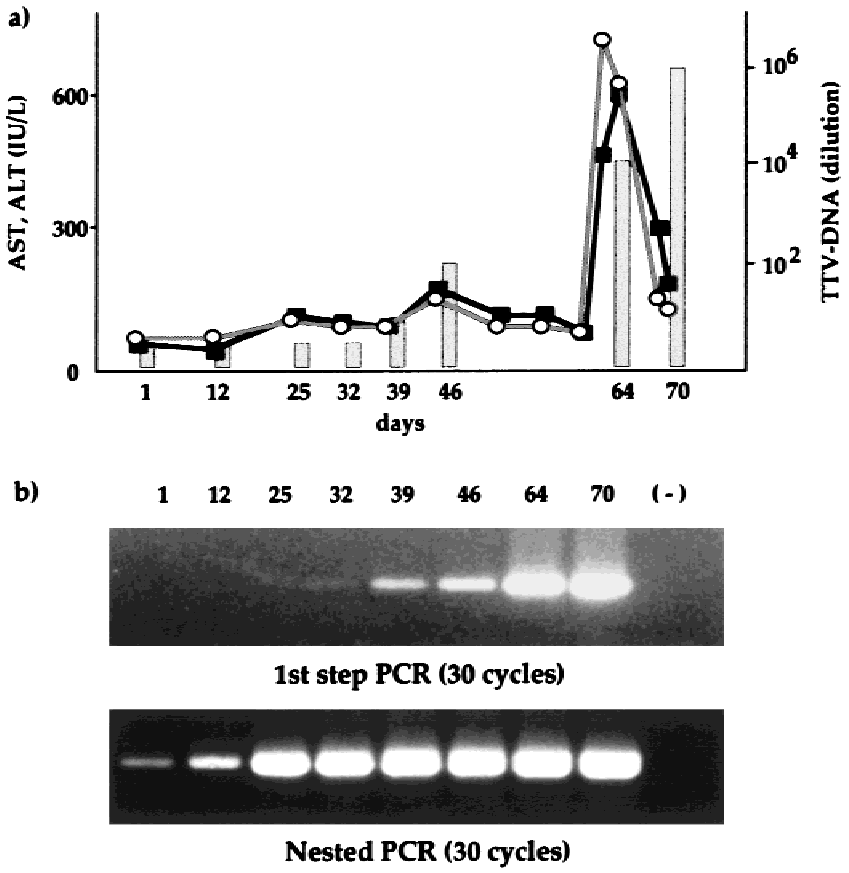

Fig. 1. Changes over time in aminotransferase levels and TTV viral load in the patient's sera. (**a**) Open circles and solid squares indicate AST and ALT levels, respectively, and vertical bars represent TTV DNA titer. Horizontal axis represents number of days since patient admission to Mizonokuchi hospital. (**b**) Amplification of TTV DNA by single step PCR and nested PCR using 100 μl of patient sera.

hepatitis markers including HAV RNA, HBV DNA, HCV RNA, HDV RNA, GBV-C/HGV RNA, and parvovirus B19 DNA were all negative by PCR.

The patient was transferred subsequently to Teikyo University Mizonokuchi Hospital on November 17, 1992, as no improvement was observed in his physical condition. Bone marrow biopsy was carried out on Day 2. The aminotransferase levels continued to be moderate, although an increase was observed on Day 63. The patient had gastrointestinal hemorrhage in addition to aplastic anemia and a general bleeding tendency, and blood and fresh frozen plasma transfusions were carried out. The patient's condition did not improve, however, and he died on January 25, 1993 (Day 70) (Fig. 1a). Autopsy was performed. Serial serum samples that had been collected from the time of admission until the patient's death were stored at −80°C. Informed consent was obtained whenever necessary, and all procedures abided by ethical guidelines established by the University.

Detection and Quantitative Analysis of Serum TTV DNA

TTV DNA was detected using the SMITEST R&D kit (Sumitomo Kinzoku Biomedical Co., Tokyo, Japan). DNA was extracted from 100 μl of patient sera, and amplification of TTV DNA was conducted by nested PCR according to the method described by Okamoto et al. [1998]. The concentration of TTV DNA in patient sera was estimated by serial 10-fold dilutions of sera, and judged by the highest dilution that yielded a PCR product.

Detection of TTV DNA in the Liver Tissue

One gram of the autopsied liver sample (that had been stored at −80°C) was minced into small pieces, washed, and lysed in 10 ml of DNA-lysis buffer (1% SDS, 10 mM EDTA/2 Na, 1 mM $CaCl_2$, pH 8.0), and digested overnight with 100 mg/ml proteinase K at 37°C with gentle agitation. DNA was extracted by phenol/chloroform/isoamyl alcohol, and precipitated by ethanol. The DNA was next treated with RNase A (Sigma, St. Louis, MO) to destroy residual RNA, and purified. Approximately 1 mg of DNA was obtained from the starting material (1 gram of liver), of which a 1 μg-aliquot of DNA (corresponding to ~1 mg of liver) was subjected to PCR for the detection of TTV DNA.

Detection of TTV mRNA in Liver Tissue

Total RNA was obtained from 1 gram of the liver autopsy material by the acid guanidinium isothiocyanate-phenol chloroform (AGPC) extraction method. mRNA was purified from total RNA using oligo-dT latex beads. Any contaminating DNA was removed by treatment with RQ I DNase (Promega Co, Madison, WI). TTV cDNA was synthesized from the pretreated

RNA by reaction with 10 units of Moloney murine leukemia virus reverse transcriptase (MMuLV-RT) (Gibco-BRL, Gaithersburg, MD) and 100 pmol of the antisense primer described by Okamoto et al., [1998]. The cDNA was subjected to nested PCR amplification. Detection of E-cadherin adhesion molecule (E-CAM) mRNA present in hepatic and bile duct epithelial cells [Rimm and Morrow, 1994], was used as the internal control reaction. Sense and antisense primers used to detect E-CAM mRNA were UV1148S (5′-TTCTACACGTAGCAGTGACGAATG-3′) and UV2744A (5′-CTAGTGGTCCTCGCCGCCTCCGTA-3′), respectively.

Southern Blot Analysis of the Liver Tissue

The possibility of TTV DNA integration into liver genomic DNA was investigated by Southern blot analysis. DNA was extracted from 1 gram of the autopsied liver by the method described above. Ten μg of the purified genomic DNA was digested overnight with 50 units of either *Bam*HI, *Eco*RI, or *Hind*III (New England Biolabs, Beverly, MA) at 37°C. The digested samples were electrophoresed on a 0.8% agarose gel. The DNA was transferred to Hybond-N (Amersham, Buckinghamshire, UK) by the alkali method [Wahl et al., 1979] and hybridized with a 2.2 kb probe constructed from the PCR product of serum TTV DNA that had been amplified using primers TTKD1S (5′-ACGCCGACCATGGAGGCGCCGCTGGAGGAC-3′) and TTKD1A (5′-TTGGTAACAAGGTAGGGTTGATATCTTGAT-3′). The probe was labeled with α-^{32}P-dCTP (Amersham) by Rigby's method [Rigby et al., 1997] using Random Labeling Kit ver. 2 (Takara Shuzo Co., Ltd., Kyoto, Japan). Hybridization was carried out at 42°C for 16 hr in a solution containing 6× SSC, 0.5% SDS, 5× Denhardt's solution, 100 μg/ml of denatured salmon sperm DNA, and 50% formamide. The filter was washed at 55°C with 0.1× SSC containing 0.1% SDS, and exposed subsequently to X-ray film (Eastman Kodak, Rochester, NY).

Detection of TTV DNA in Bone Marrow

The bone marrow samples obtained at biopsy (Day 2) and autopsy (Day 70) had been stored as formalin-fixed paraffin-embedded blocks. To test for TTV DNA, seven 10 micron-thick 1.5 cm-diameter sections (approximately 0.5 mg in total weight) were homogenized and incubated in DNA-lysis buffer at 94°C for 2 min, followed by incubation at 65°C for 30 min. Proteinase K was added to the sample solution and incubated overnight at 37. The subsequent DNA extraction and purification were carried out using methods described earlier. TTV DNA was detected by PCR in the manner described earlier for serum TTV DNA detection.

RESULTS

Detection and Quantitative Analysis of Serum TTV DNA

Serum TTV DNA was tested initially by nested PCR upon patient admission to Mizonokuchi Hospital, and was detected consistently over the course of hospitalization. Serum TTV DNA concentration at Day 1 was 10^1/ml and increased gradually to 10^6/ml by Day 70 (Fig. 1a). Between Day 1 and Day 32, serum TTV DNA was detectable only by nested (30 + 30 cycle) PCR. After Day 39, however, TTV DNA could be detected even by a single step (30 cycle) PCR procedure (Fig. 1b).

Characterization of TTV DNA in Liver Tissue by Southern Blot Analysis

DNA extracted from ~1 mg of the autopsied liver tissue was subjected to TTV DNA PCR. Nested PCR demonstrated the presence of TTV DNA in the liver tissue. To determine whether the TTV genome was integrated into hepatic cell genomic DNA, Southern blot analysis of the patient's hepatocyte DNA (10 μg) was carried out. A 2.2 kb TTV amplicon including the TTV open reading frame was used as a probe. TTV DNA could not be detected even with an extended seven-day autoradiographic exposure at −80°C.

Detection of TTV mRNA in Liver Tissue

The presence of TTV mRNA in liver tissue was examined to determine the viral replication site. Total RNA was extracted and purified from the patient liver. Pretreatment with RNase-free DNase prevented contamination by viral DNA present in the serum. Amplification of TTV DNA and the E-CAM control did not take place in the absence of reverse transcriptase, indicating minimal DNA contamination. When reverse transcriptase was added to the reaction, a 1.5 kb PCR product of the E-CAM control was obtained from both mRNA and total RNA preparations, but a predicted 290 bp product indicating the presence of TTV-derived RNA transcripts in the liver cell preparation was not obtained (Fig. 2).

Detection of TTV DNA in the Bone Marrow Tissues

To determine the presence of TTV in bone marrow cells, the formalin-fixed paraffin-embedded tissue sections (approximately 0.5 mg in weight) obtained by biopsy on Day 2 and by autopsy on Day 70 were subjected to PCR. TTV DNA was detected in both bone marrow samples, but in different amounts (Fig. 3). In contrast to serum TTV DNA levels that showed an increase from 10^1/ml on Day 1 to 10^6/ml on Day 70, PCR analysis of bone marrow indicated a higher TTV concentration at day 2 than at post mortem autopsy. The erythroblast and lymphocyte counts were also much greater in the Day 2 specimen than in the Day 70 specimen.

DISCUSSION

The patient in this study was diagnosed with subacute hepatitis of unknown etiology in association with aplastic anemia . He had received massive amounts of blood and fresh frozen plasma transfusions, and it is possible that these were the sources of his TTV infection. The serum TTV DNA test was already positive on Day 1 of hospitalization, however, before his receiving the transfusions. Thus, it is more likely that the TTV

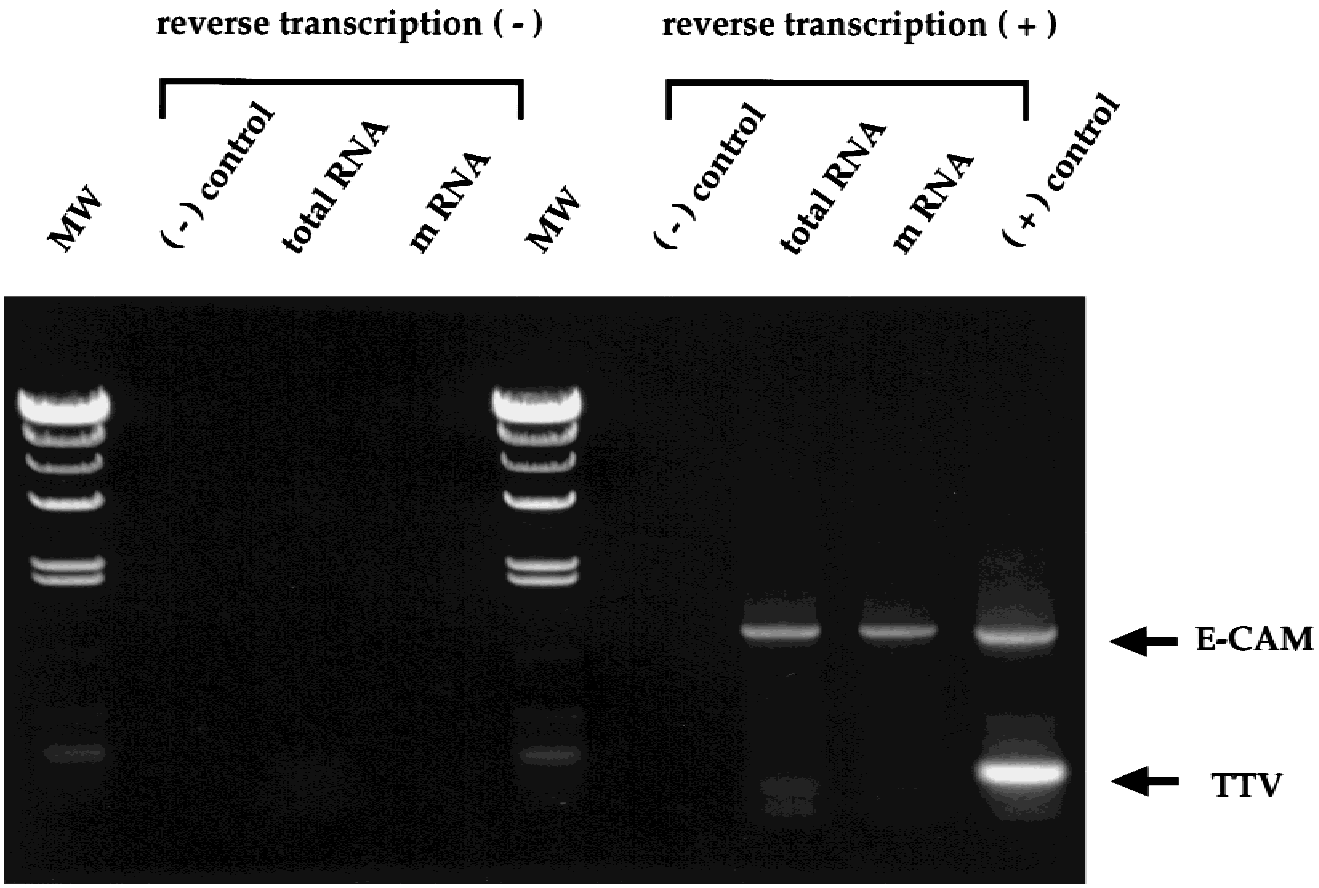

Fig. 2. RT-PCR for liver TTV-associated RNA. A TAE-agarose gel stained with ethidium bromide is shown. E-CAM mRNA was used as internal control. Amplified TTV products can be seen as bands of approximately 290 base pairs whereas those of E-CAM products can be observed at 1500 base pairs. MW is the molecular weight marker; (–) control, the negative control with no RNA added; (+) control, the E-CAM and TTV DNA cloned in plasmids.

infection occurred before the development of the hepatitis/aplastic anemia.

Although serum TTV DNA was detected in a high concentration (10^6/ml) on Day 70, only a relatively low level of TTV DNA was found in the liver. Furthermore, transcribed TTV RNA was not detected in either total RNA nor mRNA extracts from the liver (Fig. 2). Okamoto et al. [1998] have reported in a number of patients as much as a 100-fold higher concentration of TTV DNA in the liver biopsy specimen than in the serum specimen. In the present study, however, nucleic acid extracted from 1 mg of liver tissue contained only a very small amount of TTV DNA: the DNA could only be detected by a two-step nested PCR procedure. When the nucleic acid preparation was pre-treated with RNase-free DNase, neither PCR nor RT-PCR yielded any detectable TTV amplicons. Thus, it seems likely that TTV DNA detected in liver tissue was from circulating serum TTV.

Because DNA viruses are often found integrated into host genomic DNA as has been reported for hepatitis B virus [Brechot et al., 1980; Chakraborty et al., 1980; Edman et al., 1980; Shafritz et al., 1981], the possible integration of TTV DNA into the genome of hepatic cells was investigated. When a 2.2 kb TTV probe was used in a Southern blot analysis of liver genomic DNA, no signal was obtained. Thus, TTV DNA was not found to be integrated into hepatocyte chromosomes, and the liver apparently was not the site of TTV replication for this particular case. Similarly, Yamamoto et al. [1998] have reported the absence of viral replication in hepatocytes of TTV infected cases of hepatocellular carcinoma.

Interestingly, this patient's hepatitis was complicated by aplastic anemia. Members of the *Parvoviridae* family [Langnas et al., 1995] and *Flaviviridae* family [Simons et al., 1995] have been reported to suppress reticulocyte production. Parvovirus B19 has also been noted to cause fulminant liver failure and aplastic anemia [Simons et al., 1995]. Our patient was negative for serum parvovirus B19 DNA. Okamoto et al. [1998] reported a similarity in genomic structure between TTV and parvoviruses. Takahashi et al. [1998b] reported on a similarity of TTV to chicken anemia virus of the *Circoviridae* family. Results here by PCR analysis show that TTV DNA was detected at a high concentration in 0.5 mg of bone marrow biopsy specimen taken on Day 2 (Fig. 3). In 100 μl (100 mg) samples of sera taken during this period, however, TTV was present only at relatively low concentrations (<1/200 the level seen in

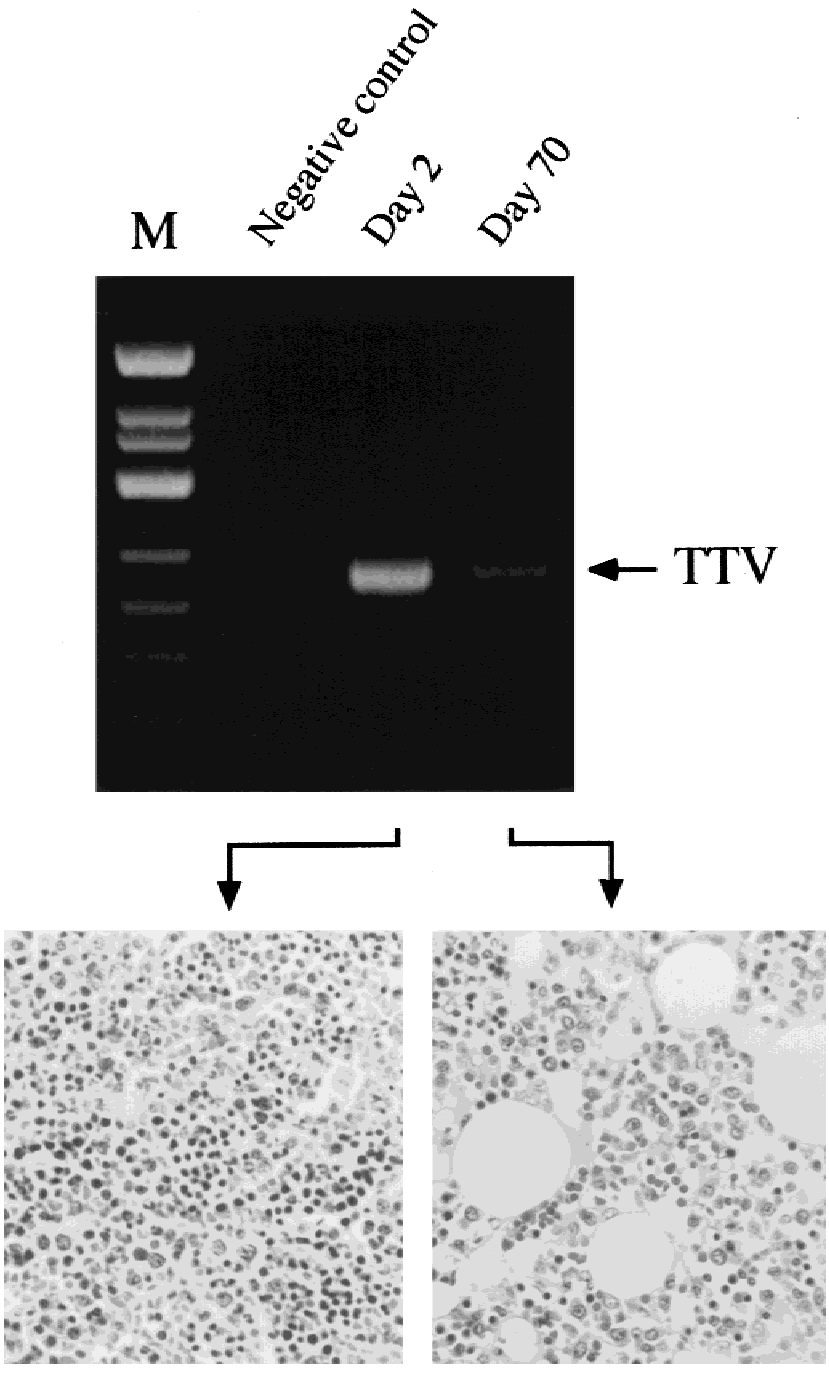

Fig. 3. TTV DNA PCR and histology of bone marrow. Single-step PCR products from the formalin-fixed paraffin-embedded bone marrow sections obtained on Day 2 and Day 70 were run on a TAE-agarose gel along with molecular size markers (M) and a negative control. Histology of the bone marrow sections (HE staining; 200×) revealed that erythroblast and lymphocyte counts differed between the Day 2 and Day 70 specimens.

bone marrow). This suggests that TTV is capable of replicating in the bone marrow as has been reported for parvovirus B19. The precise replication site for parvovirus B19 has been found to be in cells of the erythroid lineage, ranging from blast-forming unit erythroid to erythroblasts [Takahashi et al., 1990]. In this context, it is noteworthy that the biopsied bone marrow sample obtained on Day 2 yielded a much stronger TTV PCR signal than that from the autopsied sample on Day 70: the former contained a greater number of erythroblasts and also lymphocytes than the latter (Fig. 3). The difference in PCR signal strength between the Day 2 sample and Day 70 sample may be attributed differences in erythroblast or lymphocyte content in the bone marrow sample tested.

These data, taken together, suggest that the site of TTV replication in this particular patient was in the bone marrow rather than in the hepatocytes, and that TTV infection was the cause of the aplastic anemia. Similar findings suggesting TTV replication in bone marrow have been obtained by other researchers (Chayama et al., personal communication). Unfortunately, direct evidence for TTV replication in the bone marrow cells could not be established in this study be-

cause of the unavailability of mRNA from formalin-fixed paraffin-embedded specimens. In situ hybridization or immunohistology using TTV-specific probes or antibodies may provide direct evidence.

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